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Mineralization of [^{14}C]Glyphosate and its Plant-Associated Residues in Arable Soils Originating from Different Farming Systems*

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Abstract: The biomineralization of [^{14}C]glyphosate, both in the free state and as ^{14}C-residues associated with soybean cell-wall material, was studied in soil samples from four different agricultural farming systems. After 26 days, [^{14}C]carbon dioxide production from free glyphosate accounted for 34–51% of the applied radiocarbon, and 45–55% was recovered from plant-associated residues. For three soils, the cumulative [^{14}C]carbon dioxide production from free glyphosate was positively correlated with soil microbial biomass, determined by substrate-induced heat output measurement and by total adenylate content. The fourth soil, originating from a former hop plantation, and containing high concentrations of copper from long-term fungicide applications, did not fit this correlation but showed a significantly higher [^{14}C]carbon dioxide production per unit of microbial biomass.

Although the cumulative [^{14}C]carbon dioxide production from plant-associated ^{14}C-residues after 26 days was as high as from the free compound, it was not correlated with the soil microbial biomass. This indicates that the biodegradation of plant-associated herbicide residues, in contrast to that of the free compound, involves different degradation processes. These encompass either additional steps to degrade the plant matrix, presumably performed by different soil organisms, or fewer degradation steps since the plant-associated herbicide residues are likely to consist mainly of easily degradable metabolites. Moreover, the bioavailability of plant-associated pesticide residues seems to be dominated by the type and strength of their fixation in the plant matrix.

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1 INTRODUCTION

Glyphosate (*N*-[phosphonomethyl]glycine), a non-selective systemic herbicide, is widely used for the control of a great variety of annual, biennial and perennial grasses, sedges and other weeds in various crops, as well as in non-crop areas. It is classified among the less persistent pesticides.[1] In soils, it is readily mineralized to carbon dioxide, the mechanism being preferably biotic.[2–4] Its persistence and degradation vary greatly between soils. Some authors have demonstrated a positive correlation between the cumulative carbon dioxide production, resulting from mineralization, and soil respiration[5–7] or oxygen consumption[8] of soils. In contrast to numerous publications on the fate of pesticides applied to the soil in a free state, information on the degradation of plant-associated pesticide residues is limited, although the global input of plant litter containing bound pesticide residues into the soil is important. Non-extractable pesticide residues bound to plant material may be more persistent than non-bound residues in soils and may thus have long-term ecological consequences. Therefore, plant-associated residues derived from [^{14}C]glyphosate are included in the investigations reported here. Such residues have previously been characterized for wheat and soybean plants, as well as for cultured soybean cells.[9] In contrast to many of the bound pesticide residues in plants,[10] the fixation of glyphosate residues in plant material appears to be due to unspecific adsorption or to binding of the primary metabolite, AMPA, with starch and cell-wall material.[9] Therefore, the soybean preparation used in this work is referred to as 'plant-associated residues' of glyphosate rather than as 'bound residues'.

The relationship between the microbial biomass of soils sampled from different agricultural cropping systems and the mineralization of free glyphosate as well as its plant-associated residues is reported.

2 MATERIALS AND METHODS

2.1 Herbicide

[^{14}C]Glyphosate, labelled on the phosphonomethyl group, was purchased from Amersham-Buchler, Braunschweig; specific radioactivity 11·9 MBq mg^{-1}, radiochemical purity >99·7%. For the mineralization experiments, a commercial SL formulation containing 480 g glyphosate-isopropylammonium litre^{-1} ('Roundup'; Monsanto) was diluted according to the manufacturer's instructions (1 + 80, by volume) and mixed with [^{14}C]glyphosate dissolved in water, resulting in a specific radioactivity of 4767 Bq mg^{-1} active ingredient.

2.2 Soils

The soil samples used are described in Table 1. They originate from four agricultural sites under different cropping systems. The first soil, Bio (15), was taken from a site which had been cropped organically and which had received no pesticide and mineral fertilizer applications for 15 years. The second soil, Conv, emanated from a neighbouring site which had been treated regularly with pesticides and mineral fertilizers and had physicochemical properties similar to those of the first soil. The third and fourth soils had received no pesticides or mineral fertilizer for the two previous years but had different pesticide histories before that; the fourth, called 'Hop', was from a former hop plantation and had been treated regularly with copper sulfate as a fungicide, resulting in a copper concentration >200 mg kg^{-1} in the soil (Table 1).

2.3 Preparation of plant-associated residues of [^{14}C]glyphosate

Plant-associated residues of [^{14}C]glyphosate were prepared from sterile cell suspension cultures of soybean (*Glycine max* (L.) Merr. cv. Mandarin).[9,11] Fifteen flasks each containing sterile soybean cell suspension culture (40 ml) were treated with

TABLE 1
Chemical and Physical Properties of the Four Soils used in the Study

Sampling site	*Ottmaring*		*Scheyern*	
Soil	*Bio (15)*[a]	*Conv*[b]	*Bio (2)*[c]	*Hop*[d]
Clay (%)	17	16	18	13
Silt (%)	44	34	38	36
Sand (%)	39	50	44	51
pH ($CaCl_2$)	6·7	5·6	6·0	6·1
Organic carbon (%)	1·40	1·17	1·73	1·71
Total nitrogen (%)	0·15	0·12	0·17	0·18
C/N ratio	9·33	9·75	10·18	9·50
Copper content (mg kg^{-1} soil)	11	14	18	203

[a] Farmed organically over the previous 15 years, receiving no pesticides or inorganic fertilizers.
[b] Farmed conventionally, receiving pesticides and inorganic fertilizer regularly.
[c] Formerly farmed conventionally, but farmed organically over the previous two years.
[d] Formerly a hop plantation which had received regular applications of copper sulfate but farmed organically over the previous two years.

[^{14}C]glyphosate (1 μg ml^{-1}; corresponding to 265 kBq per flask) and agitated (110 rev min^{-1}) for 24 h at 27°C in the dark. The cells were filtered off, homogenized and extracted with Bligh-Dyer mixture[11] first with methanol + dichloromethane (2 + 1, by volume), then with methanol + dichloromethane + water (2 + 1 + 0·8, by volume). The insoluble residues were lyophilised and pulverized in a Dismembrator (Model II, Braun, Melsungen) for 3 min. The contents of the 15 flasks were combined and extracted three times with water, taking into account the polar nature of glyphosate and its metabolites. For the first water extraction, the residues were stirred for 16 h, and for 1 h for the second and third water extractions, at room temperature in all cases. The insoluble residues were then lyophilised, pulverized and stored at − 18°C until use.

The radioactivity associated with the insoluble glyphosate residues accounted for up to 11% of the radioactivity applied; the concentration was 7·6 nmole glyphosate equivalents g^{-1} dry weight. In former studies,[9] extraction with various solvents and solubilization with enzymes[12] showed that nearly 80% of the radioactivity which could not be extracted with the Bligh-Dyer mixture and with water was bound in the starch, protein and pectin fractions of the soybean cells.

2.4 Mineralization experiments

The mineralization of free glyphosate and its plant-associated residues was studied in a closed, discontinuously aerated laboratory system.[13] [^{14}C]Glyphosate, in the commercial formulation, was applied to the soil samples in incubation flasks, corresponding to an agricultural application dose of 2·5 kg AI ha^{-1}. Plant-associated ^{14}C-residues derived from glyphosate were then mixed with the soil in the incubation flasks (140 mg 50 g^{-1} soil). The experiments were carried out in triplicate. During 26 days, [^{14}C]carbon dioxide was collected in special traps filled with ethanolamine + diethylene glycol monobutylether (Merck, 5 + 5 by volume; 10 ml)[13] which were preceded by other traps filled with ethylene glycol monomethylether (Merck, 10 ml) for absorption of volatile organic ^{14}C-compounds.[13] At the end of the experiments, soil samples (10 g) were taken from each flask for the determination of soil microbial parameters; the remaining soil was extracted with aqueous potassium hydroxide (0·2 M) to determine the non-extractable glyphosate residues in soils.[14] Four replicate extractions were performed.

2.5 Radioactivity measurements

The radioactivity in liquid samples was determined by counting in scintillation cocktails in a liquid scintillation counter (Packard Tri-Carb 1900). Therefore, the absorption liquids in the traps containing either [^{14}C]carbon dioxide or volatile organic compounds were rinsed three times a week with scintillation cocktail (10 ml; Permablend, Packard, in toluene, 11 g litre^{-1}). The radioactivity in the potassium hydroxide, the Bligh-Dyer and the water extracts was determined by counting aliquots (500 μl) in Ultima Gold (Packard, 15 ml). The radioactivity in solid samples (dry cell residues, soil after extraction) was measured by combustion of aliquots (100–500 mg) in a Packard sample oxidizer Tri-Carb 306, followed by liquid scintillation counting of the [^{14}C]carbon dioxide evolved in Carbo-Sorb (Packard; 15 ml).

2.6 Determination of soil microbial properties

Soil microbial biomass and activity were characterized by soil heat output and by the content of adenine adenylate fractions. They were determined in each soil sample at the beginning and at the end of the mineralization experiments.

2.6.1 Total adenylate content and adenylate energy charge

The total adenylate content and the ratio of the adenylate fractions in the soil samples were determined according to Bai *et al.*[15] by extraction of the adenylates from soil, derivatization and quantification by HPLC with a fluorescence monitor. The adenylate energy charge (AEC) was calculated as follows:[16–18]

$$AEC = ([ATP] + [ADP] \times 0{\cdot}5) \times ([AMP] + [ADP] + [ATP])^{-1}$$

2.6.2 Substrate-induced heat output (SIH) and relative quotient of heat production

The basal heat output (BH; unamended soil) and the substrate-induced heat output (SIH; addition of glucose at 4 g litre^{-1}) were measured in a four-channel microcalorimeter (thermal activity monitor 2277, Thermometric, Järvalla, Sweden). The microbial biomass carbon content (C_{mic}) was calculated from the SIH according to Sparling:[19] 1 g C_{mic} [μg g^{-1} soil] = 180·05 mW. The relative heat output (*rq*heat) was used as an additional ecophysiological soil parameter describing the percentage basal heat output in relation to the substrate-induced heat production (BH [μW g^{-1} soil] × 100/SIH [μW g^{-1} soil]).[20]

2.7 Statistical evaluations

The [^{14}C]carbon dioxide production from the radiolabelled pesticide in each soil was measured in triplicate. All measurements of soil microbial parameters were carried out with at least three replicates for each soil sample. The extraction of soil samples with potassium hydroxide was replicated four times. Data were tested

by analysis of variance and the treatment means were compared by the Scheffé-test with a confidence level of 95%. Data are presented as mean values ±SE. Correlations between soil microbial parameters and the [^{14}C]carbon dioxide production from the herbicide were analyzed with Pearson correlation coefficients and Spearman, as well as the Kendall, correlation coefficients at the 95% confidence level.

3 RESULTS AND DISCUSSION

3.1 Mineralization of free [^{14}C]glyphosate and plant-associated residues of [^{14}C]glyphosate

The mineralization of free [^{14}C]glyphosate and of its residues associated with plant material is shown in Fig. 1, expressed in terms of cumulative [^{14}C]carbon dioxide as a percentage of ^{14}C initially applied. All soil samples used exhibit a high mineralization capacity both for free glyphosate and for the derived plant-associated residues. For free glyphosate, the absence of a lag phase shows that, prior to mineralization, no adaptation of the soil microflora is necessary. After about five days, the mineralization rates decrease, resulting in mineralization rates of $<1\%$ per day after 20 days. This type of curve shape is common for the mineralization of organic xenobiotic compounds in soils.[21] Free glyphosate (Fig. 1A) from the commercial formulation was mineralized best by the soil sample which had received no pesticides for 15 years, and least by the soil sample from the conventional farming system. The other two soil samples showed a medium mineralization capacity.

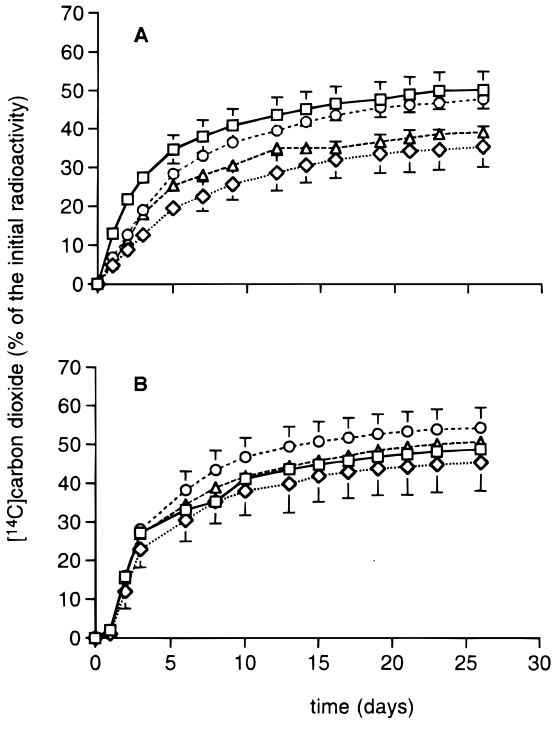

Fig. 1. (A) Cumulative [^{14}C]carbon dioxide production of ^{14}C-labelled free glyphosate (B) plant-associated glyphosate residues in samples of four soils from different agricultural farming systems during 26 days. (□) Bio (15); (○) Bio (2); (△) Hop and (◇) Conv. Vertical bars indicate ±SE from triplicates.

The mineralization of plant-associated ^{14}C-residues of glyphosate did not differ significantly among the four soil samples and showed a sigmoidal curve shape. The bioavailability of the plant-associated residues seems not to be reduced compared to that of the free herbicide; except for soil Bio (15), the mineralization rate is even greater.

For non-extractable residues of isoproturon bound in hemicellulose and lignin fractions of cell walls, the bioavailability to degrading soil micro-organisms was strongly reduced as compared to the free herbicide.[13] Glyphosate residues reported in this paper were mostly associated with starch, protein and pectin fractions of the plant cells,[9] which has little effect on their bioavailability, since these cell fractions are easily biodegradable and/or the herbicide residues are not covalently bound to the plant matrix. This demonstrates that the bioavailability of plant-associated pesticide residues is not limited by their spatial distribution, i.e. by their presence in dissolved or solid state, but is influenced by the type and strength of fixation in the plant matrix.

3.2 ^{14}C-Balance

After the incubation period of 26 days, the soil samples were extracted with potassium hydroxide solution and the liberated, as well as the non-extractable portions, were determined. The total balance of ^{14}C is presented in Table 2. The total recovery of radioactivity applied was satisfactory. The amount of volatile organic ^{14}C-compounds evolved was negligible for all soils.

The non-extractable residues (^{14}C non-extracted in soil) are formed by binding or incorporating the free herbicide or its metabolites to soil constituents. In general, this is regarded as a biotic degradation process, since it is strongly reduced in sterilized soils[22] and, for some herbicides, it is directly related to soil microbial biomass.[23] Therefore, the 'extractability of ^{14}C (^{14}C extracted as a percentage of the sum of extracted and unextracted ^{14}C) also reflects the biological degradation capacity of a soil. If this value is calculated for the four soils in this study, the 'extractability' of ^{14}C-soil derivatives from free glyphosate is significantly lower in soil Bio (15) compared to the other soils. This is in accordance with the higher biomineralization capacity of this soil.

Since the amount of soil-bound glyphosate residues was higher after the application of plant-associated glyphosate residues than after the application of free

TABLE 2
Balance of ^{14}C Radioactivity of [^{14}C]Glyphosate and its Plant-Associated Residues in Soil after 26 Days of Incubation in a Closed Laboratory System

	[^{14}C]Carbon dioxide[a]	Volatile ^{14}C-organic compounds[b,c]	^{14}C extracted with KOH[b,d]	^{14}C not extracted with KOH[b,d]	^{14}C recovery[b]
Free glyphosate					
Bio (15)	50·7	0·41	35·2	6·2	92·5
Bio (2)	48·9	0·11	45·5	6·5	101·0
Hop	39·5	1·12	42·7	4·7	88·0
Conv	34·7	1·78	48·9	6·3	91·7
Plant-associated glyphosate residues					
Bio (15)	48·8	0·11	17·9	24·9	90·8
Bio (2)	54·5	0·09	27·9	21·1	103·6
Hop	50·6	0·05	22·2	16·7	89·6
Conv	45·3	0·14	16·5	19·9	81·8

[a] Cumulative [^{14}C]carbon dioxide: SE $< \pm 10\%$.
[b] % of initial ^{14}C applied; n = 3.
[c] SE $< \pm 70\%$.
[d] SE $< \pm 15\%$.

formulated glyphosate (Table 2), this soil-bound ^{14}C is likely to be composed at least partly of non-degraded plant-associated residues. Additionally, [^{14}C]glyphosate metabolites with a high binding affinity to the soil matrix may be released from the plant-associated residues and easily immobilized in soil again.

3.3 Correlation between mineralization and soil microbial biomass

Figure 2 presents correlations between the cumulative [^{14}C]carbon dioxide production by mineralization after 26 days and the microbial biomass of the soil samples, as calculated from substrate-induced heat output. Figure 2A shows a significant positive correlation for samples of the soils Bio (15), Bio (2) and Conv (at the 95% confidence level). On the other hand, in samples of the Hop soil, there was no correlation between [^{14}C]carbon dioxide production from free glyphosate and microbial biomass. This soil, with a 200 mg kg^{-1} copper contamination (Table 1), exhibits a high mineralization capacity despite its low biomass. Therefore, the results from this soil were not included in the calculation of the correlation coefficient.

The positive correlation between mineralization and soil microbial biomass in three of the soils studied indicates that a large portion of the total microbial population in these soils contributes to the mineralization, rather than only a few highly specialized species. The exceptional behaviour of the Hop soil is due to its different microbial properties, as discussed below.

Despite the high bioavailability of plant-associated ^{14}C-residues from glyphosate, their mineralization was not significantly correlated with the microbial biomass (Fig. 2B). Similar observations have been reported for the herbicide isoproturon.[13] The mineralization of the

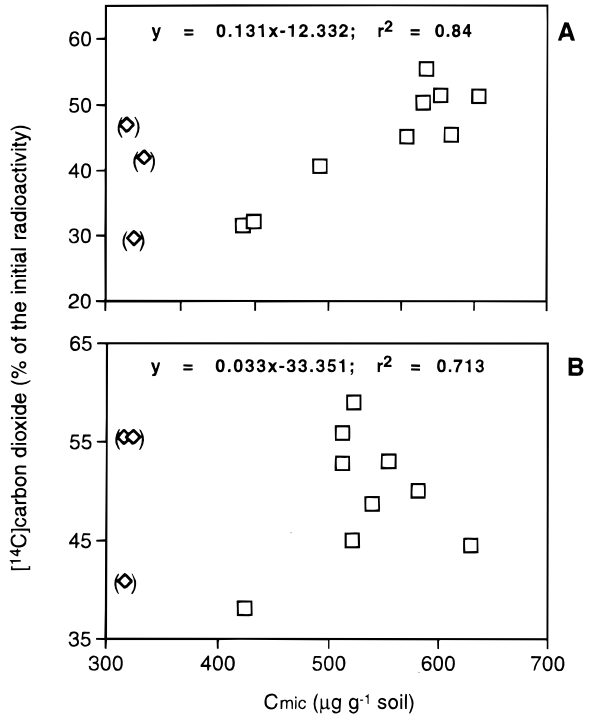

Fig. 2. Correlation between [^{14}C]carbon dioxide production from (A) ^{14}C-labelled free glyphosate and (B) plant-associated glyphosate residues after 26 days and the soil microbial biomass Cmic, estimated by SIH, in soil samples originating from four different cropping systems. Values detected in samples of the Hop soil (in parentheses) were not included in the calculation of the correlation coefficients. □ Bio (15), Bio (2) and Conv; (◇) Hop.

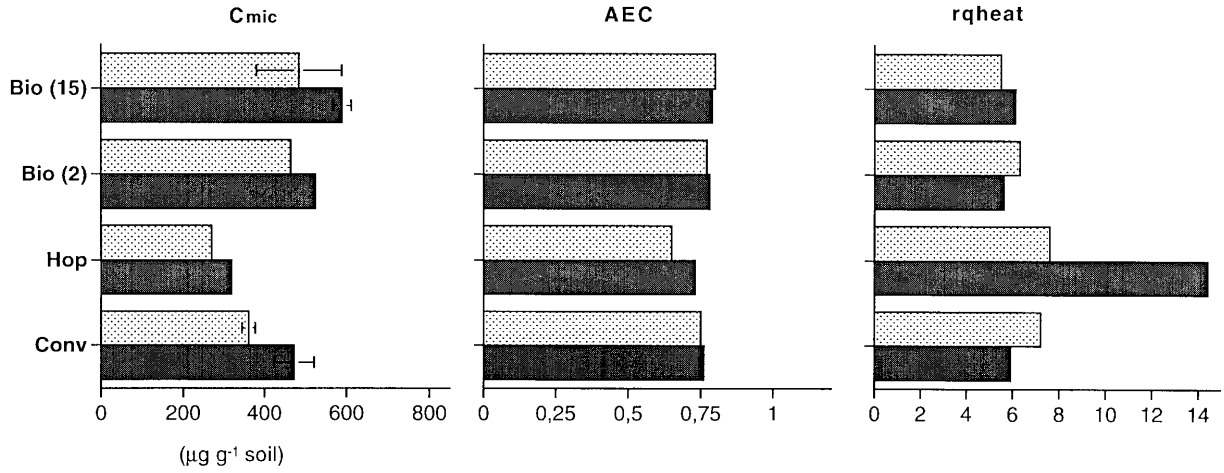

Fig. 3. Microbial biomass Cmic [μg g^{-1} soil], adenylate energy charge AEC ([ATP] + [ADP]*0·5/[AMP] + [ADP] + [ATP]) and relative quotient of heat production r*q*heat (BH[μW g^{-1} soil] × 100/SIH[μW g^{-1} soil]) of four soil samples originating from different farming systems at the end of the mineralization experiments (after 26 days) with (▨) free glyphosate and with (■) plant-associated glyphosate residues.

free herbicide was positively correlated with soil microbial biomass, while that of the plant-bound residues was not.

This suggests a different degradation mechanism for the plant-associated residues than that for the free initial compound, including more complex processes of plant matrix decomposition as well as the involvement of different groups of degrading micro-organisms, or fewer degradation steps since the plant-associated herbicide residues are likely to consist mainly of easily degradable metabolites.

3.4 Microbial activity of the soils

In addition to the total soil microbial biomass, the microbial activity was measured and compared with the mineralization rates. Figure 3 shows the soil adenylate energy charge (AEC) and the relative quotient of heat production (r*q*heat) at the end of the mineralization experiments. The AEC shows no significant differences between the soils or between free glyphosate and its plant-associated residues. By contrast, especially after the addition of plant residues, the highest r*q*heat is observed in the Hop soil. The increased ecophysiological parameter may be interpreted as a special physiological response of the microflora to the addition of organic material and consequently as an indicator for the different structure of its microbial community compared to the other three soils.

In Table 3, the [^{14}C]carbon dioxide production from free glyphosate and its plant-associated ^{14}C-residues is expressed per unit of soil microbial biomass. This compilation reveals that the microflora of the Hop soil—in accordance with its increased r*q*heat—also shows an enhanced [^{14}C]carbon dioxide production, both from free glyphosate and its plant-associated residues. The enhanced mineralization capacity of this soil, compared to the other three soils, has been reported also for the phenylurea herbicide isoproturon.[13] A probable reason for the different behaviour of the Hop soil may be its high copper content (Table 1), evoking either a physiological stress response of the organisms or a different composition of its microbial community.

TABLE 3
Quotient of [^{14}C]Carbon Dioxide Production from ^{14}C-labelled Free Glyphosate and from Plant-Associated Glyphosate Residues and Soil Microbial Biomass in Soil Samples Originating from Four Different Cropping Systems

	[^{14}C]carbon dioxide[a]/Cmic[b] Free glyphosate	*[^{14}C]carbon dioxide[a]/Cmic[b] Plant-associated glyphosate residues*
Bio (15)	0·105	0·084
Bio (2)	0·105	0·104
Hop	0·146	0·159
Conv	0·096	0·088

[a] [^{14}C]carbon dioxide represents the percentage of the initial radioactivity remaining after 26 days.
[b] Cmic in μg g^{-1} soil.

4 CONCLUSIONS

It may be concluded that the biomineralization of herbicides is positively correlated with the microbial biomass of soils originating from different agricultural farming systems, if

—a large portion of the microflora is involved in the degradation and the degrading population is ubiquitous;
—the herbicide is present in a free state; and
—the soil microflora is not impaired by long-term heavy pesticide applications.

For the mineralization of plant-associated pesticide residues, there is no obvious correlation between total decomposition of the pesticide residues and activity or content of the microbial biomass in soils, since different degradation mechanisms seem to be involved from those for the decomposition of the free compound.

The bioavailability of plant-associated glyphosate residues to degrading soil micro-organisms was not affected, whereas the bioavailability of non-extractable isoproturon residues was strongly reduced compared to the free herbicide.[13] The plant-associated residues of both herbicides differed principally between type and localization of the bonds between residues and plant matrix: plant-associated residues derived from glyphosate were associated non-specifically to the plant matrix whereas isoproturon residues were mainly bound covalently to plant cell wall fractions. Therefore, the bioavailability of pesticide residues immobilized in plant material is likely to be determined by the site and strength of the binding between plant matrix and residues, whereas their spatial distribution in soil plays only a minor role.

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